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Scientific Status Summary

# Quorum Sensing in Biofilms: Why Bacteria Behave the Way They Do

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**The Institute of Food Technologists has issued this Scientific Status Summary to provide readers with a tutorial on biofilms, their purposeful mechanism of interaction (quorum sensing), and recent findings on how to inhibit their formation.**

**Keywords: acylated homoserine lactones, autoinducer, bacteria, biofilm, *C. jejuni*, *E. coli*, enteric pathogen, foodborne illness, Gram-negative, Gram-positive, *L. monocytogenes*, pathogen, planktonic cell, quorum sensing, *Salmonella*, *S. aureus*.**

## Introduction

Even though food microbiologists often conduct experiments using planktonic cells, which are nonadherent bacteria growing as individual cells in liquid culture, biofilms are more likely to be a concern in the food industry. Human pathogens form biofilms on food and food contact surfaces, thereby enhancing their ability to survive harsh environments, resist antimicrobial treatments, and persist in the food processing environment. Normally, bacteria form complex bacterial communities that are closely associated with abiotic and biotic surfaces. These bacterial communities, known as biofilms, are adherent to a surface, an interface, or to each other. Biofilms may cause persistent low-level contamination of foods, and the presence of foodborne pathogens in a biofilm could cause food safety concerns. Cells in biofilms have been shown to detach and inoculate model food systems (Midelet and Carpentier 2004). Improvements in cleaning and sanitization have helped to reduce the persistence of these bacteria, which are more resistant to physical processes and chemical agents than their free-floating (planktonic) counterparts. In a previous Scientific Status Summary, Zottola (1994) asked whether biofilms were "a new problem for the food industry." Since that time, much has been done to understand biofilms and how to eliminate them. This Scientific Status Summary reviews the most recent research performed to understand biofilms and how to eliminate them. It begins with a basic tutorial on biofilms, explaining what they are and how they develop, and progresses to the purposeful mechanism of unified interaction that bacteria use to benefit each other: quorum sensing. The article then explains how quorum sensing processes allow bacteria to display a unified response advantageous to the population by facilitating tolerance to stress and providing access to nutrients and more favorable environmental niches. Finally, the article explores ways to prevent quorum sensing from occurring, thereby inhibiting the growth of biofilms, which may retard spoilage and benefit food production and safety.

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## Overview of Biofilms

Biofilms on surfaces have a characteristic structure consisting of microcolonies enclosed in a hydrated matrix of microbially produced proteins, nucleic acids, and polysaccharides. In this complex biofilm network, the cells act less as individual entities and more as a collective living system, often with channels to deliver water and nutrients to the cells at the inner portion of the biofilm. Biofilm organisms are significantly more resistant to environmental stresses or microbially deleterious substances (such as antibiotics and biocides) than planktonic cells. Biofilm cells present on infected tissues or medical devices are less susceptible to host immune responses than planktonic cells (Donlan 2002; Stoodley and others 2002; Sauer 2003). The gene and protein expression patterns of bacteria in biofilms, as shown by genomics and proteomics studies, differ from those of planktonic cells. Thus, the physiologies of biofilm and planktonic cells are very different (Sauer 2003).

The development of a biofilm *in vitro* involves the following 5 stages (Stoodley and others 2002):

Stage 1: reversible attachment of bacterial cells to a surface,

Stage 2: irreversible attachment mediated by the formation of exopolymeric material,

Stage 3: formation of microcolonies and the beginning of biofilm maturation,

Stage 4: formation of a mature biofilm with a 3-dimensional structure containing cells packed in clusters with channels between the clusters that allow transport of water and nutrients and waste removal, and

Stage 5: detachment and dispersion of cells from the biofilm and initiation of new biofilm formation; dispersed cells are more similar to planktonic (that is, nonadherent) cells than to mature biofilm cells.

There are a few advantages to the growth pattern of biofilms: First, bacteria are protected from the inhibitory effects of antimicrobial compounds, biocides, chemical stresses (such as pH and




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oxygen), and physical stresses (like pressure, heat, and freezing). Second, the polymeric matrix increases the binding of water and leads to a decreased chance of dehydration of the bacterial cells—a stress that planktonic cells are subject to. And third, close proximity of the microorganisms in biofilms allows nutrients, metabolites, and genetic material to be readily exchanged (Davey and O'Toole 2000; Donlan 2002; Trachoo 2003). Cell division is uncommon in a mature biofilm, and energy is used to produce exopolysaccharides, which the biofilm cells can use as nutrients (Watnick and Kolter 2000). Jefferson (2004) stated that biofilms are the default mode of growth for some bacterial species whereas planktonic growth is an *in vitro* artifact.

Biofilms usually consist of a mixed bacterial population, but they may also consist of a single bacterial species (Donlan 2002; Stoodley and others 2002). Perhaps the most obvious limitation of many of the studies performed on biofilms is that in most cases only a single organism was studied. This practice contradicts the reality that biofilms more likely consist of multiple genera and species. Lindsay and others (2002) observed interactions between *Pseudomonas fluorescens* and *Bacillus cereus* when the organisms formed binary biofilms on stainless steel. Spore counts of *B. cereus* were lower in a mixed biofilm as compared to a single-species biofilm. Moreover, treatment with chlorine dioxide was less effective against *P. fluorescens* when the biofilm also contained *B. cereus*. Interestingly, the opposite was true for *B. cereus* survival, which was lower when treated with chlorine dioxide in the mixed biofilm as compared to a single species system.

## Biofilms on Produce

The incidence of foodborne illness linked to fresh fruits and vegetables has increased significantly during the past 3 decades (Sivapalasingam and others 2004). The rise in the number of produce-related outbreaks, coupled with the lack of an effective intervention, has given rise to an intense research effort into the ecology of human pathogens in the produce production environment. Contrary to earlier theory, human pathogens have been found to survive for long periods of time in water, animal manure, and a variety of agricultural soils. Also, human pathogens have been found to be capable of attaching to and colonizing the surfaces of growing plants. It is now becoming clear that once attached, human pathogens are capable of forming biofilms on plant tissues. This formation of a biofilm has reportedly been one of the main factors in the failure of washing treatments to remove or inactivate human pathogens on produce surfaces (Annous and others 2001, 2004, 2005a).

Microscopic studies indicate that plant-associated epiphytic bacteria form biofilms on the surfaces of a wide variety of plants (Morris and others 1997; Fett and Cooke 2003). Lindow and Brandl (2003) reported that between 30% and 80% of bacteria on plant surfaces exist within biofilms. The formation of biofilms by bacteria on plant surfaces is likely a survival strategy for the cells to withstand the harsh environment of the plant surface (wide temperature changes, desiccation, ultraviolet rays, oxidative stress). Biofilm-associated bacteria embedded in a matrix of extracellular polysaccharides (EPS) might be more difficult to remove from contaminated surfaces of produce or food processing areas than their solitary counterparts (Fett and Cooke 2003; Annous and others 2004, 2005a). In addition, the production of EPS likely shields bacterial cells within biofilms from desiccation and aids in resisting antimicrobial compounds. Three commodities that have been repeatedly linked to outbreaks are cantaloupe melons, apples (as unpasteurized juice or cider), and leafy greens—each associated with a different human pathogen. Research into the interactions between *Salmonella enterica*, *Escherichia coli* O157:H7, and *Shigella* and these 3 vehicles follows.

### Cantaloupe melons

Since 1990, at least 6 multistate outbreaks of salmonellosis have been traced to the consumption of cantaloupe. The U.S. Food and Drug Administration (FDA) conducted surveys in response to a 1997 outbreak of *S. enterica* serovar Saphra which indicated that approximately 5% of imported cantaloupes tested positive for *S. enterica* (FDA 2001a). Three successive outbreaks (from 2000 to 2002) linked to the consumption of melons imported from Mexico prompted the FDA to issue an alert to detain all cantaloupes imported from Mexico and offered for entry at U.S. ports (FDA 2002).

The inability of a variety of sanitizers to remove or inactivate *S. enterica* on cantaloupes has been documented (Ukuku and Sapers 2001; Annous and others 2005a). Furthermore, the efficacy of sanitizers on cantaloupes decreased significantly when the organism was allowed to reside on the rind surface for more than 24 h. These results suggested that increased residence time allowed the formation of a biofilm prior to application of a sanitizer (Annous and others 2004). This led to investigating the ability of *S. enterica* to form biofilms on whole cantaloupe surfaces (Annous and others 2004, 2005b). Scanning electron microscopy (SEM) demonstrated that biofilm formation by *S. enterica* occurred rapidly after introduction of cells to the rind (Annous and others 2004, 2005b). Fibrillar material was visible 2 h after inoculation and drying at 20°C (Figure 1). Once attached, *S. enterica* cells developed biofilms by growth and excretion of extracellular material after 24 h of storage at either 20 or 10°C (Annous and others 2004, 2005b). Attachment and biofilm formation by *S. enterica* serovar Poona and *S. enterica* serovar Michigan inside the netting of an inoculated cantaloupe are shown in Figure 2 and 3, respectively. Cell attachment to inaccessible sites (netting) of the rind along with biofilm formation has been reported to be responsible for their resistance against aqueous sanitizers (Annous and others 2004, 2005a).

### Apples

Although 7 *E. coli* O157:H7 outbreaks associated with apple juice or cider occurred between 1982 and 2002, there have been no reports of foodborne illnesses linked to consumption of fresh apples (Rangel and others 2005). The presence of pathogens on the

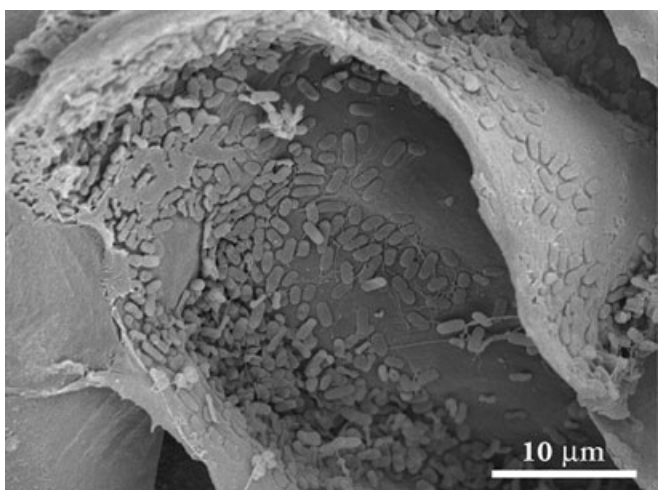


**Figure 1—Scanning electron microscopy (SEM) image showing attachment and initiation of biofilm formation by *Salmonella enterica* serovar Poona cells inside the netting of inoculated cantaloupe. Cantaloupes were inoculated and allowed to dry at 20°C for 2 h prior to imaging.**

surface of apples has implications for safety of raw material for the fresh juice and fresh-cut fruit markets. As a result, the FDA imposed a mandatory HACCP program for juice processors, which required a method that results in a 5 log reduction in the level of the target pathogen. Studies involving laboratory washing of apples—using water, detergents, or sanitizing agents—reportedly produced a maximum 3 log reduction in the levels of *E. coli* on apples (Sapers and others 2000, 2002). However, when these same apple-washing treatments incorporated the use of a commercial flatbed brush washer, there was less than 1 log reduction in *E. coli* populations (Annous and others 2001). Survival of bacteria during washing treatments was attributed to the attachment of *E. coli* cells to inaccessible regions (stem and calyx), infiltration into the calyx channel and the core of the apple, and incorporation of bacterial cells within biofilms in inaccessible sites or on the surface of the apple (Annous and others 2001; Sapers and others 2002; Fatemi and others 2006). Burnett and others (2000) demonstrated that cells attached to subsurface structures were protected against inactivation using chlorine. Annous and others (2001) reported that the majority of cells present after washing were in the stem and calyx areas. An SEM study of these areas demonstrated that *E. coli* cells were able to penetrate into the core of the apple and were able to form biofilms within the calyx region (Figure 4).

## Leafy greens

Minimally processed greens and pre-packaged salads have emerged as important vehicles of transmission for foodborne pathogens. Since 1995, the FDA has identified 18 *E. coli* O157:H7 outbreaks associated with lettuce and 1 outbreak associated with spinach. Other recent outbreaks of foodborne illness have been related to parsley, green onions, basil, cilantro, and cabbage (DeWaal and Barlow 2002; MMWR 2005). These outbreaks are accountable for hundreds of illnesses and some deaths. The sources of contamination are usually unknown, but pathogens were present in environmental samples obtained from surrounding and immediate areas of the facilities responsible for specific outbreaks (Cummings and others 2001; MMWR 2005).

Many commercial postharvest handling operations that market leafy vegetables, including the majority of packaged salad processors, effectively clean the crop surfaces with triple-wash treatments combining physical removal with various modes of disinfection. However, while planktonic bacterial pathogens are sensitive and easily controlled by relatively low levels of wash water disinfectant, the degree to which leafy products may be sanitized is highly limited and dependent on diverse intrinsic factors and the efficiencies of each commercial unit operation design and practical operation. It is well established that conventional methods of washing with hypochlorite or other sanitizing agents can achieve pathogen reduction levels of only 1 to 2 logs, which is not sufficient to ensure microbiological safety given the very low infectious dose potential of many enteric pathogens, such as *E. coli* O157:H7 (FDA 2001b; Sapers 2005). Greater reductions are not possible because of strong microbial attachment and inaccessibility due to internalization and aggregate or biofilm formation (Seo and Frank 1999; Takeuchi and Frank 2000; Solomon and others 2002). This prompted an investigation into survival, uptake, and biofilm

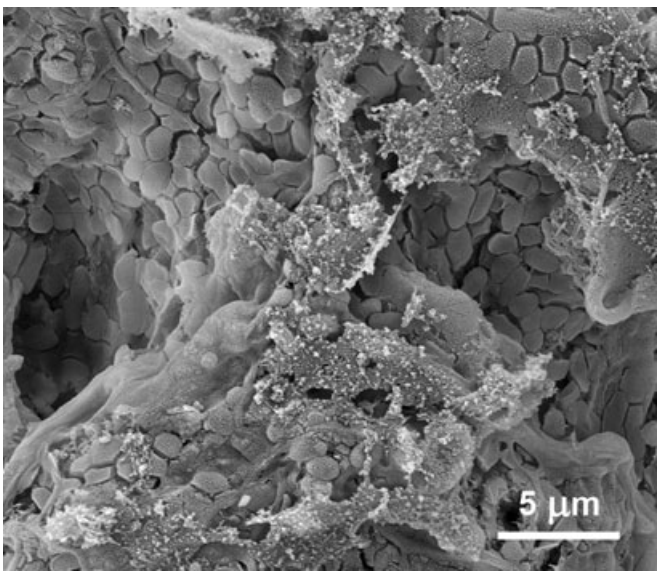


**Figure 3—SEM image showing attachment and biofilm formation by *Salmonella enterica* serovar Michigan cells inside the netting of inoculated cantaloupes after storage at 20°C for 24 h. Cells are visible within the cracks on the netting. Note the extracellular matrix encapsulating cells.**

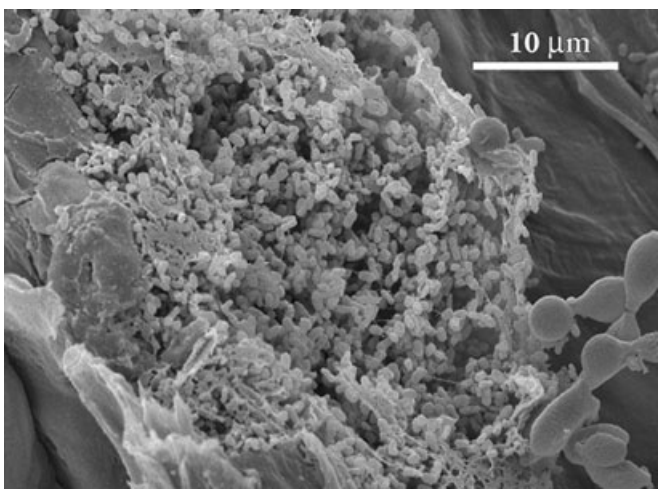


**Figure 2—SEM image showing attachment and biofilm formation by *Salmonella enterica* serovar Poona cells inside the netting of inoculated cantaloupe. Cantaloupes were inoculated and allowed to dry at 20°C for 72 h prior to imaging. Note the extracellular matrix encapsulating cells.**

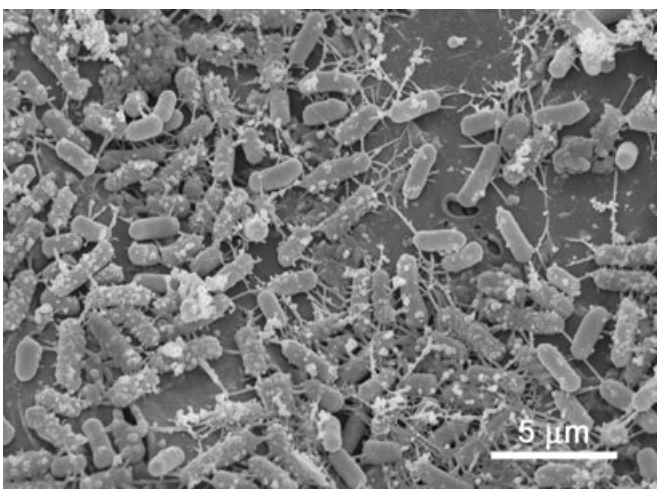


**Figure 4—SEM image showing attachment and biofilm formation by *Escherichia coli* cells in the calyx area of an inoculated Golden Delicious apple. Apples were inoculated and allowed to dry at 4°C for 72 h prior to imaging. Note the extracellular matrix encapsulating cells.**

formation by *E. coli* O157:H7 on freshly harvested lettuce leaves (Keskinen and Annous 2008). Stem ends of freshly harvested salad bowl lettuce leaves were submerged in water containing *E. coli* O157:H7 and were allowed to incubate for 1 wk at 4°C. SEM confirmed the ability of *E. coli* O157:H7 cells to survive, colonize, and form biofilm on lettuce leaves (Figure 5).

In August 1998, 8 separate outbreaks of *Shigella sonnei* occurred in 4 states and 2 Canadian provinces (Naimi and others 2003). Even though they were geographically dispersed, all of the outbreaks were linked to fresh parsley from a supplier in Mexico. In March 1999, an outbreak of *Shigella boydii* linked to bean salad prompted an investigation into the ability of *Shigella* to persist and form biofilms on the surface of parsley plants (Agle 2003). *Sh. boydii* survived well on parsley for more than 20 d when stored at refrigeration temperatures. SEM confirmed the ability of the organism to produce and become entrapped within a matrix of extracellular polymeric material on the surface of a parsley leaf (Figure 6).

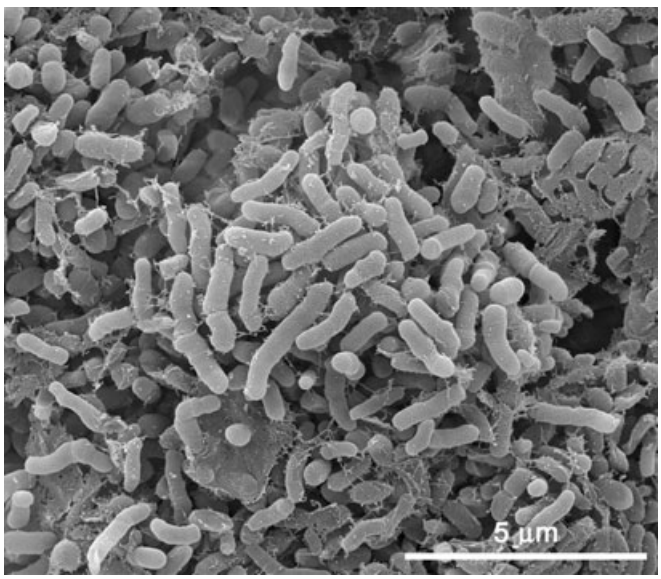


**Figure 5—SEM image showing attachment and biofilm formation by *Escherichia coli* cells on lettuce leaf. Three-week-old lettuce plants were inoculated and allowed to grow for another week prior to imaging.**

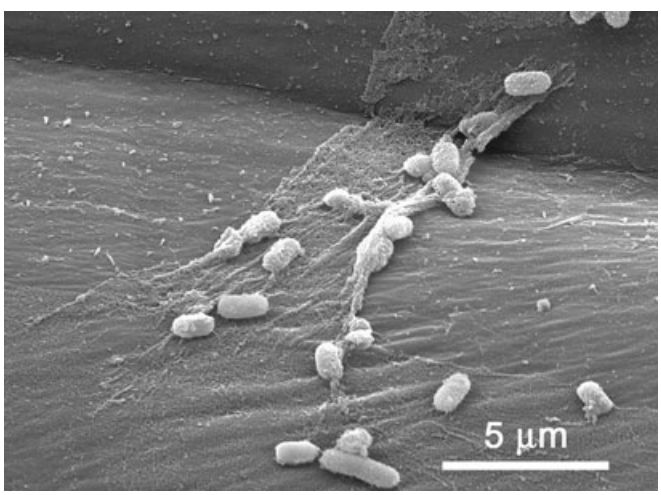


**Figure 6—SEM image showing attachment and biofilm formation by *Shigella boydii* on parsley leaf. (Image is courtesy of Scott Robinson and Meredith Agle, Univ. of Illinois.)**

These investigations into the interactions between enteric pathogens and plant tissues have documented the ability of these pathogens to form biofilms. It is likely that this phenomenon is responsible for the consistent finding that aqueous sanitizers are ineffective at inactivating human pathogens on plant tissues. New strategies that apply sanitizers and physical processes, singly and in combination, to improve their ability to overcome the protective ability of the biofilm habitat are needed.

## Overview of Quorum Sensing

Cell-to-cell signaling, known as quorum sensing, has been shown to play a role in biofilm formation in foodborne pathogens. Modulating quorum sensing processes—for example, by enzymatic degradation of the signaling molecules—will prevent biofilm formation or possibly weaken established biofilms. Bacterial gene expression in some bacterial species may be regulated by quorum sensing, a cell density-dependent signaling system mediated by chemical autoinducer molecules produced by bacteria. The autoinducer molecules bind to the appropriate transcription regulator(s) when the bacterial population reaches the quorum level (that is, the signal concentration reaches a threshold concentration sufficient to facilitate binding to the receptor). Binding of the autoinducers is followed by activation or repression of target genes. Thus, quorum sensing allows bacteria to display a unified response that benefits the population (Smith and others 2004). Bacterial quorum sensing systems enhance access to nutrients and more favorable environmental niches, and they enhance action against competing bacteria and environmental stresses. Examples of cellular processes modulated by quorum sensing are symbiosis, transfer of conjugative plasmids, sporulation, antimicrobial peptide synthesis, regulation of virulence, and biofilm formation.

There are several different quorum sensing autoinducer systems in bacteria. For example, in Gram-negative bacteria, the quorum sensing system is dependent on homologues of the *Vibrio fischeri* LuxI-LuxR regulatory proteins (Miller and Bassler 2001). Synthesized by the LuxI-like proteins, the autoinducer compounds are acylated homoserine lactones (AHLs), which are also known as autoinducer 1 (AI-1) (Figure 7). The AHLs consist of a homoserine lactone ring with a variable length acyl side chain. The AHL is synthesized inside the cell and is either diffused or secreted outside to the external environment. The concentration of AHLs increases as the bacterial population increases. When the AHLs reach a critical threshold level, they re-enter the bacterial cell to bind to the LuxR-like protein receptors. The LuxR-AHL complexes activate or repress target gene transcription (Miller and Bassler 2001).

Gram-positive bacteria also regulate a number of cellular processes through quorum sensing. However, the autoinducer compounds in Gram-positive bacteria are secreted after translation as modified peptides. Similar to AHLs, the concentration of peptides

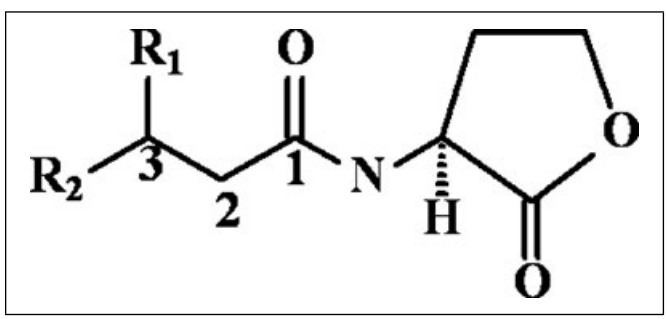


**Figure 7—Structures of acylated homoserine lactones.**

secreted to the external environment increases with the increase in bacterial populations (Miller and Bassler 2001). A major difference between the peptide signals and AHLs is that peptides generally bind to receptors on the cell surface rather than diffusing back into the cell. The peptide signals are detected by 2-component sensor kinases, and a series of phosphorylation/dephosphorylation reactions are initiated. Eventually, the process leads to the phosphorylation of the response regulator. When the phosphorylated response regulator is activated, it binds to DNA to affect transcription of the quorum sensing-controlled target gene (Miller and Bassler 2001).

A large number of bacteria have a common quorum sensing system mediated by autoinducer 2 (AI-2), which is found in both Gram-negative and Gram-positive bacteria (Figure 8). AI-2 is a product of the enzyme, LuxS, which is also involved in the activated methyl cycle (AMC) pathway and generates S-adenosylmethionine, the major methyl donor (Smith and others 2004; Vendeville and others 2005). Toxic S-ribosylhomocysteine is produced as part of the AMC pathway. One of the roles of LuxS is to detoxify S-ribosylhomocysteine by forming 4, 5−dihydroxy-2, 3-pentanedione (DPD) and homocysteine. The DPD cyclizes with boron to form AI-2. AI-2 can be considered as a byproduct of the AMC cycle (Vendeville and others 2005; McDougald and others 2007). LuxS therefore has a role in quorum sensing as well as in cellular metabolism. Boron-containing AI-2 has been shown to be involved in bioluminescence by *Vibrio harveyi* (Figure 8A) (Vendeville and others 2005; McDougald and others 2007). On the other hand, AI-2 in *S. enterica* serovar Typhimurium and *E. coli* does not contain boron (Figure 8B) (Vendeville and others 2005).

The dual role of LuxS makes it necessary to separate the metabolic role of the enzyme from the quorum sensing activity of AI-2, a product of LuxS action (Doherty and others 2006). Certain bacterial phenotypes may be due to metabolic defects owing to the loss of LuxS function in the activated methyl cycle rather than due to a defect in signaling. Therefore, to do a proper study of the effects of LuxS and AI-2, experiments must include complementation with both *luxS* gene (to discount second-site mutation effects) and purified AI-2. Such a procedure will separate the effects of AI-2 as a quorum sensing compound from metabolic effects under the control of the *luxS* gene (Hardie and others 2003; McDougald and others 2007).

Certain foodborne enteric pathogens such as *E. coli, Shigella, Salmonella*, *Yersinia*, and other Gram-negative bacterial species have the autoinducer-3/epinephrine/norepinephrine (AI-3/epi/norepi) signaling system (Walters and Sperandio 2006a). Epinephrine and norepinephrine, both mammalian hormones, cross talk with AI-3 and are recognized by the same receptor(s). Thus, there may be quorum sensing systems through which host cells communicate with bacteria (Sperandio and others 1999). AI-3 is chemically distinct from AI-2, and AI-3 synthesis is not dependent on *luxS* (Walters and others 2006). The AI-3/epi/norepi system has been shown to have an important role in the virulence of *E. coli* O157:H7 (Sperandio and others 1999; Walters and Sperandio 2006a). AI-3 activates transcription of the genes found on the locus of enterocyte effacement (LEE) chromosomal pathogenicity island in enterohemorrhagic *E. coli.* Other types of signaling molecules have been described, and these include indole; 3,4-dihydroxy-2-heptylquinolone (PQS); butyrolactones; 3-hydroxy palmytic acid methyl ester (3OH PAME); and cyclic dipeptides (Waters and Bassler 2005; Y.H. Yang and others 2005; Lee and others 2007).

*Salmonella, E. coli, Shigella,* and *Klebsiella* do not possess a member of the *luxI* family and thus do not produce AHLs. These organisms carry *sdiA* (suppressor of cell division inhibition), a LuxR homologue; therefore, they can detect AHLs produced by other bacterial species (Michael and others 2001). Several genes are regulated by *sdiA* in *Salmonella*, including *rck* found on the virulence plasmid and involved in resistance to human complement (Ahmer 2004). In *E. coli,* the *sdiA* gene cloned on a multicopy plasmid upregulated expression of genes involved in cell division, *ftsQAZ,* and in enterohemorrhagic *E. coli*, overexpression of *sdiA* caused abnormal cell division and reduced adherence to epithelial cells and expression of the intimin adherence protein (Wang and others 1991; Kanamaru and others 2000). The 5- to 13-fold upregulation of *ftsQAZ* was noted when SdiA was overexpressed on a multicopy plasmid, but *sdiA* was only slightly activated when expressed as a single copy on the chromosome compared to an *sdiA* mutant. The *sdiA* mutant did not show notable defects in cell division. Overexpressed SdiA positively regulates the multidrug resistance pump AcrAB, and it was suggested that AcrAB may play a role in the export of quorum sensing molecules (Rahmati and others 2002). The *E. coli* and *Salmonella sdiA* share only 69% amino acid identity.

Indole is formed from tryptophane by the tryptophanase enzyme and is secreted in large quantities by *E. coli* during growth in rich medium. It can act as a signaling molecule in *E. coli* and *Salmonella*, regulating the expression of a number of genes. Indole may have a role in adaptation of bacterial cells to a nutrient-poor environment in which amino acid catabolism is an important energy source. Using *E. coli* with mutations in genes that control indole synthesis, Lee and others (2007) showed that indole controls biofilm formation by repressing motility, inducing SdiA, and influencing acid resistance. They found that indole signaling decreased biofilm formation in *E. coli* while it was increased in pseudomonads. Indole and AHLs are signals in *E. coli* biofilm formation, and the mechanism of inhibition of motility and biofilm formation in *E. coli* was through SdiA.

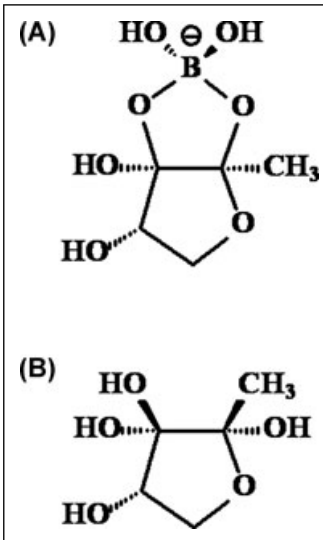


**Figure 8—Structure of auto inducer 2 (AI-2) quorum sensing molecules. (A) Boron containing AI-2 of *Vibrio harveyi*; (B) non-boron AI-2 containing AI-2 of *Salmonella enterica* serovar Typhimurium.**

## Detection and characterization of quorum sensing molecules

A number of techniques for detection and identification of quorum sensing molecules or for monitoring the activity of these compounds have been described (Swift and others 1999; Brelles-Mariño and Bednar 2001). Approaches used for detection and identification of AHLs include cell-based assays using AHL-specific bacteria biosensors, thin-layer chromatography, gas chromatography/mass spectrometry, and liquid chromatography coupled with electrospray ionization and a hybrid quadrupole linear ion trap

and Fourier-transform ion-cyclotron-resonance mass spectrometry (Ravn and others 2001; Morin and others 2003; Y.H. Yang and others 2005; Steindler and Venturi 2007; Cataldi and others 2008). Steindler and Venturi (2007) described the available bacterial biosensors used to detect various AHL signal molecules. Bacterial biosensors are mutants that cannot synthesize AHLs; however, they express a LuxR-family protein and contain an AHL-activated promoter fused to a reporter gene such as *lacZ* or *luxCDABE*. Kawaguchi and others (2008) have reported deriving a cell-free assay from the AHL-biosensor bacterium *Agrobacterium tumefaciens* NTL4, which allows the expression of beta-galactosidase upon addition of exogenous AHL. Thin-layer chromatography overlays are performed by loading plates with sample extracts or supernatants and with different standards, and after chromatography, the plates are overlaid with a soft-agar suspension containing biosensor strains. Butanloide compounds from *Streptomyces coelicolor* were detected by affinity capture with His-tagged receptor proteins and electrospray tandem mass spectrometry (Y.H. Yang and others 2005). However, genomic information on the receptor genes is necessary to harvest the quorum sensing compounds. Using the recombinant LuxR-based AHL biosensor pSB401, Holden and others (1999) detected diketopiperzine (DKP) quorum sensing molecules, which are cyclic dipeptides, in cell-free supernatants of *Pseudomonas aeruginosa, Proteus mirabilis, Citrobacter freundii,* and *Enterobacter agglomerans.* Competition studies suggested that the DKP signal molecules compete for the same LuxR-binding site as AHLs.

Determination of the presence of AI-2 in cell-free culture fluids is commonly performed using a mutant strain of *V. harveyi,* BB170 (sensor 1-, sensor 2+, luxN::Tn5), which responds to AI-2 but not to AHL (AI-1) signal molecules (Bassler and others 1994). The *V. harveyi* mutant strain, MM32, carries mutations in *luxN* and *luxS*; therefore, this strain does not produce endogenous AI-2 but possesses the ability to emit light proportional to exogenous levels of AI-2 (Kim and Surette 2006). However, DeKeersmaecker and Vanderleyden (2003) called for caution when preparing and testing cell-free culture fluids using *V. harveyi* reporter strains and use of appropriate control experiments. Their findings indicated that a time point in the range of 5 to 5.5 h after inoculation was appropriate for taking measurements for exogenous AI-2 using strain BB170 to avoid interference from endogenous AI-2 from the reporter. Furthermore, AI-2 detection was influenced by components in the growth medium and the pH of the cell-free culture fluid. Another assay method recently reported for real-time quantitative measurement of levels of AI-2 involves the use of AI-2 receptor proteins, LuxP, and LsrB, modified with environmentally sensitive dyes (Zhu and Pei 2008). Binding of AI-2 to the protein sensors produces changes in measurable fluorescence in less than 5 min in complex biological samples. Production of AI-3 has been detected using transcriptional fusions of LEE gene promoters with a *lacZ* reporter introduced into the chromosome of an *E. coli* K12 strain (Sperandio and others 1999).

Quorum sensing peptides are usually present in low amounts in complex mixtures, so they are difficult to detect and identify. A multistage mass spectrometry approach using a novel matrix-assisted laser desorption/ionization-quadrupole ion trap mass spectrometer was applied for rapid detection and characterization of peptides secreted by microorganisms (Kalkum and others 2003). The generated fragmentation signatures enable unambiguous identification of peptides of interest and differentiation against background signals. Autoinducing peptides (AIP) from supernatants of *Staphylococcus aureus* were identified, and the native group III-AIP was determined a nonapeptide (RIPTSTGFF).

## Role of Quorum Sensing in Biofilm Formation

There is evidence that in some bacteria, biofilm formation is a carefully orchestrated process controlled by quorum sensing. The use of bacterial strains with mutations in genes involved in the production of signaling molecules and the analysis of temporal differential gene expression in biofilms are revealing information on the molecular mechanisms of biofilm formation and the role of quorum sensing. While most research supports the role of quorum sensing in biofilm formation and in the resulting characteristics of the biofilm community, few studies indicate that quorum sensing does not affect the formation of biofilms. Moreover, knowledge of the chemical structures of different types of signaling molecules allows the identification of compounds that can be used to modulate quorum sensing-related processes, including biofilm formation. Additional research is needed to understand how quorum sensing works mechanistically in biofilms and how cell-to-cell signaling may influence the virulence and antimicrobial resistance of biofilm communities. This information is important to identify possible targets and to design strategies that control biofilm formation on industrial, medical, and food and food processing surfaces. Studies examining the role of cell-to-cell signaling systems in biofilm formation in foodborne pathogens are as follows.

***Campylobacter jejuni.*** *C. jejuni* is a spiral or spirochete, rod-shaped Gram-negative microaerophilic bacterium that can cause gastroenteritis and is commonly associated with foodborne illness. The production of AHLs has not been established in *C. jejuni* (Smith and others 2004). The enzyme LuxS was found in *C. jejuni*, and an AI-2-like product has been demonstrated (Cloak and others 2002; Elvers and Park 2002; Jeon and others 2003, 2005; Reeser and others 2007); however, chemical characterization of these AI-2-like compounds has not been done. Cloak and others (2002) demonstrated the production of AI-2 activity in milk and chicken broth by both *C. jejuni* and *C. coli* and have sequenced the *luxS* gene in both organisms.

Utilizing the M129 strain of *C. jejuni*, Reeser and others (2007) demonstrated that a mutation in the *luxS* gene led to a 3.2-fold decrease in biofilm formation when compared to the isogenic wild-type strain. Addition of sterile culture supernatant (24-h growth) from the wild-type strain M129 led to an increase in biofilm formation. Unfortunately, Reeser and others (2007) did not determine whether the culture supernatant contained AI-2 nor did they add chemically synthesized AI-2 to the mutant. Thus, it is not clear if the decrease in biofilm formation shown by *luxS* was due to a defect in cellular metabolism or a lack of AI-2.

The examination of Gram-negative isolates from a vegetable-processing facility revealed that their ability to form biofilms was not dependent on AHL or AI-2 production (Van Houdt and others 2004). Similarly, *C. jejuni*, a foodborne pathogen associated with poultry, was capable of forming biofilms on surfaces used in animal production watering systems (Reeser and others 2007). Mutants lacking the *luxS* gene, which is responsible for AI-2 production, formed biofilms to a lesser extent than the wild-type strain.

***Aeromonas hydrophila.*** *A. hydrophila* is a Gram-negative rod and facultative anaerobe present in all freshwater environments and in brackish water. Some strains of *A. hydrophila* are capable of causing illness in fish and amphibians as well as in humans, who could acquire infections through open wounds or by ingestion of a sufficient number of the organisms in food or water. *A. hydrophila* also causes opportunistic infections. The formation of mature biofilms on stainless steel coupons by *A. hydrophila* required the production of $C_4$-HSL since an *ahyI* (AHL synthase) mutant lacking the ability to form $C_4$-HSL did not produce a mature biofilm. A mutation in the *ahyR* (AHL receptor) gene had no

effect on biofilm formation (Lynch and others 2002). An unidentified LuxR-like receptor bound the AHL to produce biofilm in the *ahyR* mutant. Thus, Lynch and others (2002) have shown that quorum sensing regulates biofilm formation, and a number of researchers have shown that mutations in polar and lateral flagella formation decrease biofilm formation in *A. hydrophila* (Gavin and others 2002; Altarriba and others 2003; Canals and others 2006a, 2006b). Although studies have not been reported, it is probable that flagella formation in *A. hydrophila* is regulated via quorum sensing.

***Helicobacter pylori.*** *H. pylori* is a microaerophilic helical-shaped Gram-negative bacterium found in the stomach and duodenum. It has been associated with chronic gastritis, gastric ulcers, and stomach cancer. The organism can survive in low-acid environments and in refrigerated conditions, and it can be present in food and water, which can cause human infection. A strain of *H. pylori* with a mutation in *LuxS* gene showed an approximately 3-fold increase in the formation of biofilms on glass surfaces as compared to the wild-type strain (Cole and others 2004). The *luxS* gene apparently exerted a negative control on biofilm formation in *H. pylori.* However, Cole and others (2004) did not address the possible role of AI-2 in biofilm formation.

***Bacillus cereus.*** *B. cereus* is a rod-shaped facultative aerobic Gram-positive bacterium that forms endospores and has been associated with foodborne illness. The quorum sensing system of *B. cereus* consists of PlcR (the *plcR* gene encodes a transcriptional regulator) and PapR (the *papR* gene encodes a cell-to-cell signaling peptide) as well as the LuxS/AI-2 system (Slamti and Lereclus 2002, 2005; Auger and others 2006). A *plcR*-negative mutant produces approximately 4-fold more biofilm on polystyrene than its isogenic wild-type parent (Hsueh and others 2006). The addition of exogenous AI-2 to wild-type *B. cereus* (containing the LuxS system) decreased biofilm formation (Auger and others 2006). Also, the addition of AI-2 to established biofilms of *B. cereus* led to detachment of cells from the biofilm (Auger and others 2006). Thus, PapR and AI-2 had a negative effect on biofilm formation by *B. cereus.*

***Listeria monocytogenes.*** *L. monocytogenes* is a facultative intracellular pathogenic Gram-positive coccoid rod-shaped bacterium and the cause of listeriosis. It is associated with foods such as milk, cheeses (particularly soft-ripened varieties), ice cream, raw vegetables, fermented raw-meat sausages, raw and ready-to-eat meat and poultry, and raw and smoked fish. Resistant to the injurious effects of freezing, drying, and heating, it is able to grow at temperatures as low as 3°C. Biofilm formation by *L. monocytogenes* has been demonstrated on polyvinyl chloride microtiter plates, glass slides, stainless steel, polyethylene, teflon coupons, conveyer belt materials (such as polypropylene, acetal, stainless steel), and floor drains of food processing facilities (Zhao and others 2004; Chae and others 2006; Pan and others 2006; Lemon and others 2007; Rodríguez and McLandsborough 2007; Tolvanen and others 2007).

While *L. monocytogenes* has a LuxS/AI-2 system, the *luxS* gene was shown to repress biofilm formation (Sela and others 2006). A mutation in the *luxS* gene resulted in a 4-fold thicker biofilm than in the wild type, and the addition of *in vitro* synthesized AI-2 to cultures of the mutant did not repress biofilm formation (Challan Belval and others 2006). Therefore, there is no indication that there is a quorum sensing role for AI-2 in *L. monocytogenes* biofilm formation.

Little is known about possible peptide quorum sensing compounds in *L. monocytogenes.* However, recent data indicate that *L. monocytogenes* has an accessory gene regulator (*agr*) system. There are 4 genes, *agrB, agrD, agrC,* and *agrA,* in the *agr* operon (Rieu and others 2007). There is an approximately 62% decrease in the number of cells attached to glass slides with *agrA* and *agrD* deletion mutants of *L. monocytogenes* as compared to the wild type (Rieu and others 2007). In addition, these mutants showed a 33% decrease in the amount of biofilm formed on polystyrene during the first 24 h; however, at 48 and 72 h, the amount of biofilm formed by the mutants and wild type was approximately the same. The early delay in biofilm formation was probably due to decreased adhesion of the cells to the plastic surface (Rieu and others 2007).

***Escherichia coli.*** *E. coli* is a facultative anaerobic Gram-negative rod-shaped bacterium that causes gastrointestinal and extra-intestinal infections. *E. coli* serotype O157:H7 causes hemorrhagic colitis and hemolytic uremic syndrome, and outbreaks have been linked to contaminated water and foods such as ground beef, raw milk, and produce. *E. coli* strains produce biofilms on the surfaces of glass, stainless steel, high density polyethylene, polyamide-6, polyvinyl chloride, teflon coupons, glass wool, polystyrene microtiter plates, and glass coverslips (Faille and others 2002; Hancock and Klemm 2007). The LuxS/AI-2 system is present in *E. coli* (Ahmer 2004; Walters and Sperandio 2006b). Enzymatically synthesized AI-2 increased motility and stimulated the formation of biofilms when added to wild-type *E. coli* K-12 strains (González Barrios and others 2006). However, Yoon and Sofos (2008) found that an AI-2 producing strain of *E. coli* O157:H7 and its isogenic non-AI-2-producing mutant behaved similarly in terms of biofilm formation on solid surfaces. Thus, the role of the LuxS/AI-2 system in biofilm formation by *E. coli* is still not clear.

An isogenic *sdiA*-negative mutant of *E. coli* K-12 demonstrated a several-fold increase in biofilm formation as compared to the wild-type strain. The wild-type K-12 showed a decrease in biofilm formation if AHLs were added; the *sdiA* mutant did not respond to AHLs (Lee and others 2007). Thus, *E. coli* K-12 responds to exogenous AHLs with a decrease in biofilm formation, and this decrease requires SdiA.

Mutations that led to decreased levels of extracellular and intracellular levels of indole in *E. coli* K-12 led to increased motility and biofilm formation, which indicates that indole inhibits biofilm formation (Domka and others 2006; Lee and others 2007). A decrease in motility and biofilm formation resulted from the addition of indole to the wild type or to the mutants. The addition of 600 $\mu$M indole induced *sdiA* almost 3-fold as determined by microarray studies. A deletion in *sdiA* enhanced both motility and biofilm formation, indicating that SdiA protein represses motility and biofilm formation. Indole added to an *sdiA* mutant had little effect on biofilm formation but did decrease motility (Lee and others 2007). In the wild-type *E. coli* K-12, short-chain AHLs (in the presence of low levels of indole) led to reduced biofilm formation; *sdiA* mutant did not respond to AHLs. Lee and others (2007) suggested that indole may inhibit biofilm formation by binding to SdiA.

***Salmonella enterica.*** *S. enterica* consists of a large number of different serovars that are associated with foodborne and waterborne gastroenteritis. They are facultative anaerobic Gram-negative rod-shaped bacteria. Biofilms were formed by *S. enterica* on polystyrene microtiter plates by 15 clinical isolates (6 serovars), by 26 produce-related isolates (19 serovars), and by 31 meat-related stains (15 serovars) (Solomon and others 2005). *S. enterica* serovar Enteritidis formed biofilms at the air/liquid interface on stainless steel coupons and glass slides (Niemira and Solomon 2005; Giaouris and Nychas 2006). *S. enterica* serovar Typhimurium DT104 formed biofilms on polyvinyl chloride microplates, stainless steel and glass surfaces, and polystyrene microtiter plates (Ngwai and others 2006; Kim and Wei 2007). In the presence of bile, *S. enterica* serovar Typhi and serovar Typhimurium formed biofilms on the surface of gallstones (Prouty and others 2002).

Thus, biofilm formation appears to be a common trait in *S. enterica* serotypes.

The LuxR homologue in *Salmonella* species is SdiA; however, they do not have the LuxI homologue and therefore cannot synthesize AHLs (Walters and Sperandio 2006b). In addition, LuxS/AI-2 is present in *Salmonella* (Walters and Sperandio 2006b). A *luxS* mutant of *S. enterica* serovar Typhimurium does not form a mature biofilm on polystyrene (De Keersmaecker and others 2005). Biofilm formation was restored when the mutant was complemented by a functional *luxS* gene, but there was no biofilm formation on addition of 4,5-dihydroxy-2,3-pentanedione (DPD, precursor to AI-2; the compound has been shown to have AI-2 activity) (De Keersmaecker and others 2005). However, Yoon and Sofos (2008) found that biofilm formation was similar in AI-2 positive and negative strains. Accordingly, the relationship between biofilm formation and the presence of an active LuxS system and AI-2 in *S. enterica* is not clear.

***Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica*.** The yersiniae are facultative anaerobic Gram-negative short rods. *Y. pestis* causes bubonic plague, but *Y. enterocolitica* and *Y. pseudotuberculosis* are associated with foodborne illness. *Y. pestis* and *Y. pseudotuberculosis* strains formed biofilms on glass or polystyrene (Joshua and others 2003; Patel and others 2006). *Y. enterocolitica* formed biofilms on granular activated carbon columns, but reports of biofilm formation on other abiotic surfaces by *Y. enterocolitica* are not available (Camper and others 1985). There are 2 *luxI*/*luxR*-like genes in *Y. pestis*, *yspI*/*yspR* and *ypeI*/*ypeR* (Kirwan and others 2006). The AHL synthase YspI synthesizes mainly N-3-oxo-octanoyl-L-homoserine lactone (3-oxo-$C_8$-HSL) and N-3-oxo-hexanoyl-L-homoserine lactone (3-oxo-$C_6$-HSL) in approximately 1:1 ratio (Kirwan and others 2006). The *ypeI*/*ypeR* system has not been characterized. Similarly, *Y. pseudotuberculosis* has 2 *luxI* synthase genes: *ypsI* and *ytbI* (Ortori and others 2007). At least 24 different AHLs are produced by wild-type *Y. pseudotuberculosis*. In *Y. enterocolitica*, YenI directs the synthesis of 3-oxo-$C_6$-HSL and $C_6$-HSL as well as smaller amounts of 3-oxo-$C_{10}$-HSL, 3-oxo-$C_{12}$-HSL, and 3-oxo-$C_{14}$-HSL (Atkinson and others 2006). Medina-Martínez and others (2006) demonstrated that *Y. enterocolitica* produced AHLs in milk and in liquid extracts of beef, fish, and pork; however, it did not produce AHLs liquid extracts of mixed lettuce, cucumber, or soy bean. Jarrett and others (2004) demonstrated the presence of the *luxS* gene in *Y. pestis*, and it is likely that the gene is also present in *Y. pseudotuberculosis* and *Y. enterocolitica*.

Swarming motility is a flagella-dependent movement of bacteria in the presence of extracellular slime, allowing bacteria to spread over a surface; it is distinct from swimming motility. Swarming has been implicated in the formation of biofilms (Harshey 2003; Daniels and others 2004). It has been demonstrated that swimming and swarming motility are controlled by *yenI* in *Y. enterocolitica*, and swarming motility is implicated in biofilm formation (Harshey 2003; Daniels and others 2004; Atkinson and others 2006). While *yenI* is necessary for swimming and swarming and probably biofilm formation in *Y. enterocolitica*, the role of AHLs is still not clear.

***Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*.** Members of the genus *Vibrio* are facultative anaerobic Gram-negative curved rod-shaped bacteria that are associated with foodborne and waterborne diseases. *V. cholerae* causes cholera whereas *V. vulnificus* is associated with wound infections, enteritis, bacteremia, and death in immunocompromised individuals. A number of studies have examined quorum sensing in vibrios; the most recent was a review by Milton (2006).

*V. cholerae* formed biofilms at the air/surface interface of glass tubes and on glass coverslips, glass beads, and polyvinyl chloride microtiter plates (Hammer and Bassler 2003; Zhu and Mekalanos 2003; Joelsson and others 2006; Fong and Yildiz 2007). *V. parahaemolyticus* formed iofilms on glass coverslips and polystyrene microtiter plates (Güvener and McCarter 2003; Enos-Berlage and others 2004; Shime-Hattori and others 2006). In addition, *V. parahaemolyticus* formed pellicles at air/liquid interfaces (Güvener and McCarter 2003; Enos-Berlage and others 2004). *V. vulnificus* formed biofilms on the surfaces of glass tubes, glass coverslips, and polystyrene (Joseph and Wright 2004; Lee and others 2004; Paranjpye and Strom 2005; McDougald and others 2006). As a wound pathogen, the organism may also form biofilms in human tissue.

Biofilm formation in *V. cholerae* is tightly regulated and controlled by multiple quorum sensing systems operating simultaneously to regulate the transcription of genes involved in the production of exopolysaccharide. The organism forms biofilms at low (rather than high) cell densities when signal molecules have accumulated (Hammer and Bassler 2003). Furthermore, exopolysaccharide-overproducing variants readily arose during the time course of the biofilm assay, trapping smooth parental cells within the biofilm. Mutations in *hapR*, a transcriptional regulator, were responsible for enhanced biofilm formation. This suggests that at low cell densities or early in the infection, it may be advantageous for *V. cholerae* to be able to form biofilms and express virulence genes. At higher cell densities, the pathogen loses its ability to adhere and form biofilms, permitting escape from the host into the external environment. HapR is a negative regulator of biofilm formation in *V. cholerae*, and its expression is induced at high cell densities. By monitoring the expression of *hapR*, Z. Liu and others (2007) have shown that quorum sensing is activated earlier and at higher levels in biofilm rather than planktonic cells and that timing of *hapR* expression is important for controlling biofilm thickness, detachment rates, and colonization efficiency.

The LuxS/AI-2 system has been found in *V. parahaemolyticus* (Henke and Bassler 2004; Defoirdt and others 2006). *V. parahaemolyticus* synthesize AHLs via the LuxM synthase and the AHLs are sensed by LuxN (a 2-component sensor) (McCarter 1998; Henke and Bassler 2004). The presence of the LuxS/AI-2 system has been demonstrated in *V. vulnificus*, as well as the LuxR homologue, SmcR (McDougald and others 2001; Shao and Hor 2001; Kim and others 2003). A number of AHLs have been isolated from cultures of *V. ulnificus* including $C_4$-, $C_6$-, 3-oxo-$C_8$-, 3-oxo-$C_{10}$-, 3-oxo-$C_{12}$-, and 3-oxo-$C_{14}$-HSL, which indicates that a synthase must be present (Morin and others 2003).

A *smcR* (a *luxR* homologue) mutant of *V. vulnificus* produced an approximate 5-fold increase of biofilm on polystyrene as compared to the wild type (McDougald and others 2001, 2006). The effect of LuxS/AI-2 on biofilm formation by *V. vulnificus* has not been reported. Thus, the role of quorum sensing on biofilm formation by *V. parahaemolyticus* is still not known.

***Staphylococcus aureus*.** *S. aureus* is a Gram-positive coccus, which on microscopic examination appears in pairs, short chains, or grape-like clusters. Some strains are capable of producing highly heat-stable enterotoxins capable of causing staphylococcal food poisoning. *S. aureus* strains form biofilms on polystyrene and glass microtiter plates as well as on Teflon catheters and other medical devices (Gross and others 2001; Götz 2002; Beenken and others 2004). Biofilm formation by *S. aureus* is associated with human and animal infections. Biofilm-associated infections of humans include endocarditis, osteomyelitis, skin infections, and others (Yarwood and Schlievert 2003).

Quorum sensing in *S. aureus* is based on the secretion of a short peptide attached to a 5-membered thiolactone ring (autoinducing peptide [AIP]) encoded by *agr* locus. Attachment of the secreted AIP to the AIP cell surface receptor initiates a series of phosphorylation reactions culminating in the activation of the transcription regulator, RNA-III, which initiates transcription of the targeted genes (Otto 2001, 2004). Expression of *agr* controls virulence in staphylococci (Yarwood and Schlievert 2003; Kong and others 2006). Four AIP subgroups have been found in *S. aureus*; the AIP of 1 subgroup inhibits expression of the *agr* regulon of the other subgroups, but growth is not inhibited. AIP activates the virulence response in strains of its own subgroup and represses the *agr*-mediated virulence response in other AIP subgroups (Otto 2001; Smith and others 2004).

The LuxS/AI-2 system is present in *S. aureus* (Doherty and others 2006; Kong and others 2006). Inactivation of the *luxS* gene in *S. aureus* did not affect the *agr*-dependent AIP signaling system, and a mutation of *agr* had no effect on LuxS (Doherty and others 2006). Researchers found that a mutation in the *luxS* gene of *S. aureus* had no effect on virulence-associated traits or biofilms (on polystyrene microtiter plates) or the *agr* signaling system. Apparently, the *luxS* gene had a role in metabolism of the cell but was not involved in quorum sensing.

Vuong and others (2000) studied 105 strains of *S. aureus* and found that 78% of strains lacking the *agr* locus (21/27) produced biofilms on polystyrene whereas only 6% (5/73) of $agr^+$ strains produced biofilms. The addition of *S. epidermidis* AIP to the *agr* mutant of *S. aureus* led to a 5-fold decrease in biofilm formation. Therefore, an *agr* mutant demonstrates increased biofilm formation as compared to the wild type.

## Genetic Transfer within Biofilms

The frequency of gene transfer in planktonic cells is probably lower than that seen in cells found within biofilms (Roberts and others 2001). A number of studies indicate that transfer of genes is a common phenomenon in biofilms (Lebaron and others 1997; Dahlberg and others 1998; Licht and others 1999). In single species biofilms on glass beads, a strain of donor *E. coli* harboring 3 different plasmids transferred (probably by conjugation) the plasmids to an *E. coli* strain present as a biofilm (Lebaron and others 1997). A rifampicin-resistant strain of *E. coli* (recipient) was allowed to form biofilms on glass, and at 8 d, a donor strain of *E. coli* carrying the plasmid R1*drd*19 (confers resistance to chloramphenicol and ampicillin) was added to the biofilm. Within 24 h, rifampicin-resistant transconjugants with resistance to chloramphenicol and ampicillin were isolated (Licht and others 1999). Horizontal gene transfer by transformation was demonstrated in strains of *Streptococcus mutans* by Li and others (2001). Maeda and others (2006) used 2 F-minus (nonconjugative) *E. coli* strains (one strain with a chromosomal tetracycline resistance gene and the other with a nonconjugative plasmid and a kanamycin resistance gene) to study gene transfer in biofilms. In mixed biofilms of the 2 *E. coli* strains on nylon membrane filters, cells with resistance to both antibiotics were detectable. Gene transfer in biofilm cells at 25 °C was approximately 1 log higher than that of planktonic cells. The mechanism of gene transfer was probably transformation (Maeda and others 2006). Using DNA from *V. cholerae* O139 added to a chitin biofilm of *V. cholerae* O1 El Tor suspended in artificial sea water, Blokesch and Schoolnik (2007) found transformants with the characteristics of the O139 strain. In a mixed biofilm of O1 El Tor (rifampicin resistant and streptomycin sensitive) and O139 (rifampicin sensitive and streptomycin resistant), transformants with O139 characteristics and rifampicin resistance and streptomycin sensitivity were isolated. In the presence of deoxyribonuclease, no transformants were present, thereby indicating that transformation was the gene transfer mechanism (Blokesch and Schoolnik 2007). It is apparent from the literature that the transfer of antibiotic resistance genes is common in the biofilm environment. The results obtained by a number of researchers indicate that gene transfer occurs readily in biofilms by either conjugation or transformation. In addition, data also support that genetic exchange occurs at a higher frequency in biofilm cells than in planktonic cells.

## Genomic and proteomic analysis of biofilm formation

Genomic- and proteomic-based techniques such as DNA microarrays or 2-dimensional gel electrophoresis allow analysis of the metabolic pathways that contribute to growth and survival of pathogens in foods, the food processing environment, and in humans. These techniques also enhance the understanding of biofilm formation at the molecular level but have not revealed a common gene and/or protein expression pattern for biofilm formation in microorganisms (Ghigo 2003; Sauer 2003). Sauer (2003) stated that biofilm cells differ from planktonic cells in their patterns of gene expression (and probably protein expression). Using an extraintestinal pathogenic *E. coli* strain that formed biofilms on urinary catheters, Orme and others (2006) demonstrated that outer membrane protein A (OmpA) was upregulated in the strain as compared to a laboratory noninfectious strain of *E. coli*. They suggested that upregulation of OmpA may be a common feature of urinary tract-infectious *E. coli*; thus, OmpA could be a potential therapeutic target for the inhibition of biofilms on urinary catheters. Trémoulet and others (2002) did a proteomic analysis of planktonic and biofilm cells of *E. coli* O157:H7 and found that the levels of 14 proteins increased and those of 3 proteins decreased in biofilm cells as compared to planktonic cells. Upregulated proteins in biofilm cells included DNA protection during starvation protein, D-ribose-binding periplasmic protein, D-galactose-binding protein, malate dehydrogenase, amino acid ABC transporter-binding protein, thiamine-phosphate pyrophosphorylase, and DNA-binding protein H-NS. Li and others (2007) examined the role of *lsrR* and *lsrK*, 2 genes involved in the uptake of AI-2 in *E. coli*, on quorum sensing-related functions. Based on data using *lsrR* and *lsrK* mutants and microarray analyses, Li and others (2007) suggested that AI-2 may bind with LsrR to affect biofilm formation and architecture by regulating interactions among genes that control biofilm formation, including genes involved in colonic acid synthesis and phase variable protein antigen 43. In addition, they found that small RNAs (sRNAs) interact with quorum sensing regulators in *E. coli* and thus affect biofilm formation.

Proteomic analysis of biofilm forming *C. jejuni* indicated that proteins involved in motility were upregulated in comparison with planktonic cells (Kalmokoff and others 2006). Proteins involved in motility, including the flagellins (FlaA and FlaB), the filament cap (FliD), the basal body (FlgG and GlgG2), and the chemotactic protein (CheA); proteins involved in general (GroEL, GroES) and oxidative (Tpx, Ahp) stress responses; adhesins (Peb1 and FlaC); and proteins involved in biosynthesis, energy generation, and catabolite functions were upregulated in biofilm-forming cells.

In a proteomic comparison of *L. monocytogenes* (serotype 1/2a) cells grown planktonically and in biofilms formed on nitrocellulose membranes, Hefford and others (2005) found that 19 proteins were upregulated in biofilm-grown cells. These proteins included glyceraldehyde 3−phosphate dehydrogenase, triose phosphate isomerase, phosphoglycerase mutase, chaperon GroEL (a stress protein), and elongation factor EF-Tu (involved in the transportation of

aminoacyl-tRNAs to the A site of the ribosome during elongation) (Hefford and others 2005).

Ren and others (2005) used microarrays to study gene expression in *E. coli* K12 in the presence and absence of ursolic acid, a compound that inhibits biofilm formation. Nineteen genes were consistently induced with exposure to ursolic acid, including genes involved in chemotaxis, motility, and heat shock response. Twelve genes were consistently repressed, including genes involved in cysteine synthesis and sulfur metabolism. Thus, ursolic acid may inhibit biofilm formation by causing an increase in cell motility. Microarray results also indicated that sulfur metabolism through *cysB* affects biofilm formation in the absence of ursolic acid. Furthermore, ursolic acid inhibited biofilm formation without interfering with quorum sensing because there was no effect on the level of AI-1 or AI-2 activity in cells exposed to usolic acid. In another study, DNA microarrays were used to study differences in gene expression in *E. coli* wild-type cells and *yliH* and *yceP* mutants to identify genes involved in biofilm formation (Domka and others 2006). Other reports had shown that these 2 genes were induced in biofilms (Schembri and others 2003; Ren and others 2004). Domka and others (2006) showed that YliH and YceP are involved in the regulation of indole transport, export of AI-2 through a cAMP-dependent pathway, and the stress response. They proposed that *yliH* and *yceP* be renamed *bssR* and *bssS*, respectively, for "regulator of biofilm through signal secretion" (Domka and others 2006).

## Interfering with Quorum Sensing

Countermeasures to cell-to-cell signaling have been explored in an attempt to reduce the ability of cells to form biofilms, attenuate virulence, and modulate other processes influenced by quorum sensing. Inhibition of quorum sensing can be accomplished in several ways, which include (1) enzymatic degradation of the signal molecule, (2) blocking signal generation, and (3) blocking signal reception (Hentzer and Givskov 2003; Roche and others 2004; Kjelleberg and others 2008).

AHLs present in bacterial cultures are degraded nonenzymatically at pH values above 7 (Byers and others 2002; Yates and others 2002; Flodgaard and others 2003). The degradation of AHLs at alkaline pH values is due to lactonolysis—that is, opening up of the lactone ring through hydrolysis of the ester bond of the ring to give an acylhomoserine (Yates and others 2002). AHLs also can be degraded enzymatically. A number of bacteria produce lactonases that hydrolyze the ester bond of the homoserine lactone ring (Roche and others 2004; Dong and Zhang 2005; Rasmussen and Givskov 2006). In addition, a few bacteria have been shown to degrade AHLs through acylase action, cleaving the amide bond connecting the lactone ring to the acyl chain, releasing the homoserine lactone and fatty acid, which may be further metabolized by the bacteria (Roche and others 2004; Dong and Zhang 2005; Rasmussen and Givskov 2006; D. Liu and others 2007).

AHL lactonases have been demonstrated in mammalian sera (F. Yang and others 2005) and an AHL acylase (acylase I) has been identified in a porcine kidney (Xu and others 2003). *Bacillus megaterium* cytochrome P450 can oxidize AHLs and acylhomoserines (produced by lactonase action) (Chowdhary and others 2007). Insertion of a *Bacillus* species AHL lactonase gene into tobacco and potato plants led to protection of plants against the AHL produced by the plant pathogen *Erwinia carotovora*, demonstrating the feasibility of the use of AHL lactonases as antimicrobial agents (Dong and others 2001).

Halogenated furanones produced by the marine alga *Delisea pulchra* are antagonistic toward AHL-controlled processes (de Nys and others 1993; Givskov and others 1996) (Figure 9). The halogenated furanones prevent binding of AHLs to the *luxR* homologues, resulting in the rapid turnover of these proteins (Manefield and others 2002). The brominated furanone, (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone (Figure 9A), of *Delisea pulchra* interfered with quorum sensing mediated by both AHL (AI-1) and AI-2 (Ren and others 2001). The furanone inhibited swarming of *E. coli* and also inhibited biofilm formation, as evidenced by a decrease in thickness of approximately 50%, a reduction in water channels, and a reduction in the number of live cells. This furanone also inhibited AI-1- and AI-2- mediated quorum sensing in *V. harveyi*. The action of AHL is inhibited by the furanone through destabilization of LuxR; however, it is not clear how the brominated furanone inhibits AI-2 action (Ren and others 2001). Other brominated furanone compounds (Figure 9B and 9C) have also been shown to inhibit quorum sensing (Smith and others 2004).

AHL signaling is involved in biofilm formation by *P. aeruginosa* since a mutant of *lasI*, which is a gene that encodes synthesis of AHLs, does not produce a mature biofilm (Davies and others 1998). Hentzer and others (2002) demonstrated that brominated furanones had an adverse effect on the architecture (that is, interference with maturation) of *P. aeruginosa* biofilms and enhanced the detachment of bacteria from the biofilm.

AI-2 promotes biofilm and swimming motility in *E. coli* (González Barrios and others 2006). Ren and others (2001, 2004) found that swarming activity and biofilm formation by *E. coli*

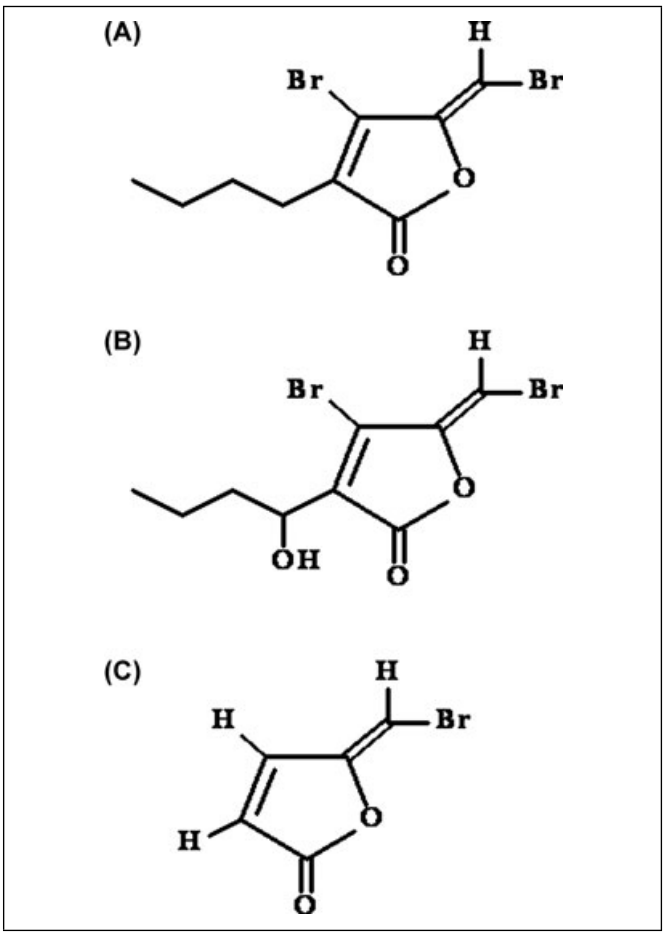


**Figure 9—Structures of 3 brominated furanones. (A) 4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone; (B) 4-bromo-5-(bromomethylene)-3-(1, hydroxybutyl-2(*5H*)-furanone; (C) 5-(bromomethylene)-2(*5H*)-furanone.**

were inhibited by (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2-(5H)-furanone (Figure 9A) at levels that had no effect on bacterial growth; however, swimming was not inhibited. The brominated furanone decreased the concentration of AI-2 but had no effect on *luxS* and *pfs* genes, which encode the proteins for AI-2 production (Ren and others 2004).

Other compounds produced by eukaryotes that are capable of interfering with bacterial quorum sensing systems have been described (González and Keshavan 2006). These include L−canavanine, which is an arginine analog found in the seeds of legumes and in penicillic acid and patulin and is produced by fungi belonging to the genus *Penicillium*. Methods have been described for identification of novel compounds that inhibit quorum sensing, such as using reporter systems fused to quorum sensing-controlled promoters (Kjelleberg and others 2008). Another strategy is to design compounds that interfere with quorum sensing by creating AHL analogs that have modifications in the side chain or the ring moiety (Persson and others 2005; Kjelleberg and others 2008).

Niu and others (2006) found that cinnamaldehyde partially inhibited transcription induced by AHL and decreased bioluminescence in 2 different *Vibrio harveyi* reporter strains that respond to AHL and AI-2, respectively. Thus, cinnamaldehyde affected both intraspecies and interspecies quorum sensing. Foods such as beef and turkey patties, chicken breast, and beef steak inhibit AI-2 activity (Lu and others 2004). Inhibition of AI-2 activity has also been shown for certain food additives such as sodium propionate, sodium benzoate, and sodium acetate (Lu and others 2004).

Quorum sensing may also influence spoilage in some food products. Spoilage of bean sprouts inoculated with an AHL mutant strain of *Pectobacterium* was delayed compared to sprouts inoculated with the wild type (Rasch and others 2005). Quroum sensing regulated 4 phenotypes: pectinase, protease, cellulose activities, and siderophore-mediated iron chelation. Using a bean sprout model system in a subsequent study, several compounds, including various AHL analogs, patulin, and penicillic acid, recognized as quorum sensing inhibitors, did not prevent spoilage due to *Pectobacterium* even though protease activity of the bacterium was decreased in a broth system using some of the AHL analogs (Rasch and others 2007). It was suggested that quorum sensing inhibitors must be used in the specific system in which they were tested, and one cannot assume that they will function in a similar manner in a different quorum sensing system.

Rasmussen and others (2005) constructed quorum sensing inhibitor selectors (QSIS) for screening for quorum sensing inhibitors and identified 4-nitro-pyridine-N-oxide and garlic extracts as the 2 most active inhibitors. When garlic extract, shown to have inhibited quorum sensing in *P. aeruginosa*, was added to a biofilm of *P. aeruginosa*, cells in the biofilm became more sensitive to the antibiotic tombramycin compared to nongarlic treated biofilms (Bjarnsholt and others 2005). Mice with pulmonary infections due to *P. aeruginosa* that were treated with garlic extract exhibited increased clearing of the infection as compared to untreated infected mice. The compound responsible for the activity of the garlic extract was not identified. Persson and others (2005) also identified products isolated from garlic as inhibitors of quorum sensing likely through competitive binding with LuxR, and these compounds did not have antimicrobial properties. The most potent quorum sensing inhibitor from garlic was *N*-(heptylsulfanylacetyl)-L-homoserine lactone.

Foods may contain AI-2- and AHL-like activity or compounds that mimic or inhibit autoinducer activity (Lu and others 2004). In studies examining produce, Lu and others (2004, 2005) observed AI-2-like activity in several types of fruits, vegetables, and frozen fish. Although the observed activity could be due to indigenous microbial populations on the food products or to nonmicrobial components of the food, Lu and others (2004) quantified bacterial populations on some of the products and found no correlation between observed AI-2-like activity and the number of bacteria present. Subsequently, Lu and others (2005) suggested that AI-2-like activity may increase the likelihood of biofilm formation on the products. To further study this, they examined the ability of a non AI-2 producing mutant *E. coli* to form biofilms with and without exposure to rinses from tomatoes. Biofilm formation significantly increased when the mutants were exposed to the tomato rinse (Lu and others 2005).

## Conclusion

It is becoming increasingly evident that quorum sensing enhances the ability of bacteria to access nutrients or more favorable environmental niches and to increase bacterial defenses against eukaryotic hosts, competing bacteria, and environmental stresses. The physiological and clinical aspects of quorum sensing have received considerable attention and have begun to be studied at the molecular level. However, little is known about whether quorum sensing plays an important role in food spoilage, biofilm formation, or on growth and/or toxin production of pathogens present in food. Additional research is needed to understand the mechanism(s) of biofilm formation in foodborne pathogens and the influence of cell-to-cell signaling. Clearly, various genes and pathways are involved in biofilm formation in different bacteria; furthermore, various quorum sensing systems are present in different bacteria. There has been relatively little work to date to understand quorum sensing in food systems compared to clinical settings. Some studies that have been conducted indicate that certain food components may affect quorum sensing, and signaling molecules produced by bacteria in food may affect the behavior of microorganisms within that environment. Questions that should be addressed include the following:

- Does the food environment play a role in initiating or inhibiting quorum sensing responses?
- How does food or the food processing environment affect quorum sensing and the resultant production of biofilms?
- Outside of the implications on biofilms, do spoilage and pathogenic organisms communicate in foods or in food processing environments?

Use of proteomic and genomic techniques should help to elucidate the phenotypes associated with quorum sensing and the mechanisms by which these pathways are activated or repressed. A number of compounds have been isolated or synthesized that antagonize quorum sensors, and the application of these antagonists may potentially be useful in inhibiting growth, virulence mechanisms, and/or biofilm formation of bacteria in different environments, including food. With a greater understanding, it may become possible for foods to be formulated to interfere with quorum sensing and thus inhibit growth of spoilage or pathogenic organisms, virulence, and biofilm formation, which would greatly benefit food production quality and safety.

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